CORRELATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) AND Ki-67 EXPRESSION WITH HISTOLOGICAL GRADE AND STAGE OF RENAL CELL CARCINOMA

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ABSTRACT

BACKGROUND

Renal cell carcinoma (RCC) is the most common malignant kidney tumour in adults. The vascular endothelial growth factor (VEGF) is thought to play a major role in tumour angiogenesis. Ki-67 has been shown to be of prognostic significance in RCCs. With the availability of more effective molecular targeted therapy for specific renal neoplasms, immunohistochemical (IHC) techniques play an important role in diagnosis and prognostication of RCCs. This study aims to evaluate the expression pattern of VEGF and Ki-67 in histologically diagnosed cases of RCC and correlate and compare VEGF and Ki-67 expression with grade and stage of RCC and also compare VEGF expression and Ki-67 index if any.

MATERIALS AND METHODS

This was a cross-sectional observational study. A total of 50 patients of RCC undergoing total and partial nephrectomy were included in this study. The expressions of Ki-67 and VEGF were studied by IHC. Statistical Analysis was performed with the help of Epi Info (TM) 7.2.2.2. p value of ≤0.05 was considered statistically significant.

RESULTS

Out of 50 tumours, 21 tumours were VEGF Grade 1, 19 tumours were VEGF Grade 2. 27 tumours had Ki67 labelling index≥15%. There was significant association of VEGF grades and Ki-67 labelling index with stage and histological grade. Ki-67 labelling index showed significantly increasing trend with the increase in VEGF grades of the tumours.

CONCLUSION

This study suggests that there are significant differences in VEGF expression and Ki-67 labelling index with tumour stage and grade and other prognostic parameters of RCC. These biomarkers can be considered as a prognostic parameters and critical evaluator of targeted chemotherapy in RCC. A wider evaluation involving large number of cases with proper follow up facility is needed to validate these findings.

KEY WORDS

RCC; VEGF; Ki-67

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BACKGROUND

Renal cell carcinoma (RCC) is the most common malignant kidney tumour in adults. Worldwide, it is the 9th most common cancer in men and 14th in women.[1] The case fatality rate is lower in highly developed countries than in countries with low or medium levels of socioeconomic development. It is the 3rd leading cause of death among urologic tumours and is resistant to chemotherapy and radiotherapy.[2-3] However, there has been a huge development in effective molecular targeted therapies in past few years for specific types of RCC with different histology and molecular abnormalities.

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Therefore, accurate histological diagnosis and classification have become increasingly important in these cases.[4] Among renal cell tumours, common malignant varieties are Clear Cell Renal Cell Carcinoma (CCRCC), Papillary Renal Cell Carcinoma (PRCC) and Chromophobe Renal Cell Carcinomas (ChRCC). Usually histological diagnosis of renal tumours can be done easily by routine Haematoxylin and eosin (H&E) stain. However, immune markers have become essential in several contexts which include differentiating renal and non-renal neoplasm and sub-typing of RCCs.[5] Symptoms of all types of kidney tumours are very similar and nonspecific. Most common is the triad of symptoms including painless hematuria, palpable mass or abdominal lump and flank pain.[1] But different histologic subtypes are known to have distinct prognosis. It has been very challenging to predict the prognosis of each of the patients with RCC. Classic prognostic factors, staging and grading were also not always very helpful.[6] So there has been a definite need for better tools in predicting the clinical course of RCC in this era of molecular targeted therapies.

Angiogenesis is important in determining tumour progression and development of metastases.[7] Vascular endothelial cell growth factor (VEGF) is one of the key players of angiogenesis and it has a role in promoting proliferation, survival, and migration of endothelial cells. VEGF helps in new vessel formation in tumours by acting as a chemoattractant for bone marrow derived progenitor cells.[8-9] It has been documented in numerous studies that higher VEGF-A levels correlate with high vascular density, higher proliferation rate, higher nuclear grade and advanced tumour stage resulting in poor clinical outcome.[10] Recent developments in our understanding of the molecular pathways controlling tumour angiogenesis have led to the development of novel VEGF-targeting agents for treatment of RCCs.[11]

Proliferation index as determined by Ki-67 is known to be of prognostic importance and is known to correlate with tumour grade in RCCs. Ki-67 is a non-histone protein that is usually found in all the phases of cell cycle (G1, S, G2 and mitosis) while it is absent in nondividing cells (G0).[12] This property makes it an excellent marker for determining the proliferating activity of tumour cells.

Here we performed an institution-based study on nephrectomy specimens which were histologically diagnosed as RCC and applied IHC to correlate VEGF and Ki-67 expression with grade and stage of RCC and compare VEGF expression and Ki-67 labelling index, if any and also discussed their possible utility as tissue-based biomarkers.

MATERIALS AND METHODS

Study Design

Cross-sectional observational study.

Specimens and General Information

A total of 50 specimens of RCCs were studied which were collected from patients undergoing total and partial nephrectomy in the department of Urosurgery of a tertiary care institute in eastern India from February 2017 to July 2018. Sample size was taken based on the convenience of the study. It was a cross-sectional observational study. Histologically diagnosed RCCs were included in the study and renal tumours other than RCCs were excluded from the study.

Histopathological Examination

Grossing and reporting of total and partial nephrectomy specimens with RCCs were done according to CAP (College of American Pathologists) protocol[13] which is based on AJCC/UICC TNM, 8th edition.[14] Specimens were fixed in 10% neutral buffered formalin. Representative areas were sampled, and histopathological examination was done following proper tissue processing, paraffin embedding and staining with haematoxylin-eosin (H&E) respectively. After histological confirmation of diagnosis of RCC, following parameters were analysed: histological type, tumour grade, lymphovascular and perineural invasion, necrosis, sarcomatoid or rhabdoid differentiation and staging. Specific histologic subtype was assigned according to WHO 2016 classification of tumours of the Urinary System and Male Genital Organs[1]. Histologic grading of tumours was done according to WHO/ International Society of Urological Pathology (WHO/ISUP)[1] grading system for CCRCC and PRCC. Chromophobe carcinomas could not be graded using this system. (Figure 1) Pathologic (pTNM) staging was done according to American Joint Committee on Cancer 2010.[14]

Immunohistochemistry (IHC) for VEGF and Ki-67

Immunohistochemistry was performed on 3µ sections taken on poly-L-Lysine coated slides. Primary antibodies which were used for detection of VEGF and Ki-67 are as follows-

Ki-67: Monoclonal Mouse Anti-Human, RTU, clone MIB-1, Novocastra, Leica.

VEGF: Monoclonal rabbit antibody, RTU, clone: RBT-VEGF, Bio SB. Di-amino benzidine (DAB) was used as chromogen. Positive controls which were used are as follows- (1) Lobular capillary haemangioma for VEGF (2) Tonsil for Ki-67. Negative control was achieved by omitting primary antibody.

Evaluation of IHC Staining:

For quantitative analysis of Ki-67, first hot spots were determined using low power and then approximately 1000 cells were counted in 5 high power fields. Only nuclear staining was considered positive and staining intensity was not assessed. Ki-67 index was expressed as percentage of positive staining cells among total number of invasive cells in the area scored. We used our own laboratory cut-off value of 15% according to Mehdi MZ et al.[15] For VEGF, staining was determined semiquantitatively according to a three-grade scale according to Yildiz E et al[16]:

- 0: no staining of tumour cells;
- 1+: membranous stain with no cytoplasmic immunostaining or with light cytoplasmic staining of some tumour cells (<50%);
- 2+: diffuse and strong membranous and cytoplasmic staining of most tumour cells (>50%).

Statistical Analysis

Statistical Analysis was performed with help of Epi Info (TM) 7.2.2.2 which is a trademark of the Centers for Disease Control and Prevention (CDC). Using this software, basic cross-tabulation and frequency distributions were prepared. $\chi 2$ test was used to test the association between different variables under study. Corrected $\chi 2$ test was used in case if any one of cell frequency was found less than 5 in bivariate frequency distribution. T-test was used to compare two means. Diagnostic accuracy, sensitivity, specificity, positive predictive value and negative predictive value were calculated to compare the findings of different diagnostic tools. $p \leq 0.05$ was considered statistically significant.

RESULTS

A total of 50 cases of renal cell carcinoma were studied. The average age of patients at the time of diagnosis was estimated to be 52.52±9.32 years with range 35 – 73 years and the median age was 51 years. Most of the patients (72.0%) were of in age group 40 – 59 years which was significantly higher than other age group ($p<0.001$) of which 88.0% patients were males, 68.0% patients having a history of cigarette smoking. 60.0% patients were hypertensive and 52.0% were obese. 60.0% of cases had right sided disease.

According to WHO/ISUP[1] grading system, 12.5% of the tumours were grade 1, 33.3% grade 2, 25.0% grade 3 and 29.2% grade 4. There were 20.0% cases of stage I, 28.0% cases of stage II, 50.0% cases of stage III and 2% cases of

stage IV disease respectively. Necrosis was found among 62.0% cases. 18.0% cases showed sarcomatoid differentiation whereas 4.0% cases showed rhabdoid differentiation. Among the 50 cases, 22 (44.0%) showed microscopic evidence of lymphovascular space invasion (LVSI), and 6 (12.0%) presented with microscopic evidence of perineural invasion (PNI).

Distribution of Renal Cell Carcinomas According to Histological Diagnoses and Expression of VEGF and Ki-67 in Various Sub-Types

Out of the 50 cases studied, 43 cases were CCRCC, 5 cases were PRCC and 2 cases were ChRCC. For CCRCC, most of the tumours (44.2%) were VEGF Grade-1 with 41.9% Grade-2 tumours and 14% of Grade-0 tumours. For ChRCC one tumour was negative for VEGF (grade 0) and one tumour was VEGF grade 1. No case with Grade-2 was found. For PRCC the VEGF grades were Grade-0 in most of the cases (60.0%). (Figure 2)

For CCRCC, Ki-67 labelling index was ≥15% in most of the cases (60.5%). For ChRCC all the cases were with Ki-67 labelling index<15% (100.0%). For PRCC, Ki-67 labelling index was <15% in most of the cases (80.0%). (Figure 3)

There was no significant association between VEGF grades and histological types of RCC (p=0.10). Ki-67 labelling index showed no significant association with histological type of tumours. (p=0.067)

Correlation of VEGF Grade with Grade and Stage of RCCs:

Out of 10 stage-I tumours, 8 cases showed VEGF expression as grade 0, 1 case showed VEGF expression as grade 1 and 1 case showed VEGF expression as grade 2. Out of 14 stage-II tumours, 2 cases showed VEGF expression as grade 0, 8 cases showed VEGF expression as grade 1 and 4 cases showed VEGF grade 2. Out of 25 stage-III tumours, no case showed VEGF expression as grade 0, 12 cases showed VEGF expression as grade 1 and 13 cases showed VEGF expression as grade 2. We found only one case of stage IV tumour which was VEGF grade 2. So, we found that VEGF grades showed significantly increasing trend with the increase in stage of the tumours, which was statistically significant (p<0.00001).

Out of 6 Grade 1 tumours, 4 cases showed VEGF expression as grade 0, 2 cases showed VEGF expression as grade 1 and no case showed VEGF expression as grade 2. Out of 16 grade 2 tumours, 5 cases showed VEGF expression as grade 0, 11 cases showed VEGF expression as grade 1 and no case showed VEGF expression as grade 2. Out of 12 grade 3 tumours, no cases showed VEGF expression as grade 0, 5 cases showed VEGF expression as grade 1 and 7 cases showed VEGF expression as grade 2. Out of 14 grade 4 tumours, no cases showed VEGF expression as grade 0, 2 cases showed VEGF expression as grade 1 and 12 cases showed VEGF expression as grade 2. So, it was noted that VEGF grades showed significantly increasing trend with the increase in histological grade of the tumours, which was statistically significant. (p<0.00001). (Table 1)

Correlation of Ki-67 Labelling Index with Grade and Stage of RCCs:

Out of 10 stage-I tumours, 9 cases showed Ki-67 labelling index <15% and 1 case showed Ki-67 labelling index ≥15%. Out of 14 stage-II tumours, 6 cases showed Ki-67 labelling index <15% and 8 cases showed Ki-67 labelling index ≥15%. Out of 25 stage-III tumours, 8 cases showed Ki-67 labelling index <15% and 17 cases showed Ki-67 labelling index ≥15%. We found only one case of stage IV tumour which showed Ki-67 labelling index ≥15%. So, it was noted that Ki-67 labelling index showed significantly increasing trend with the increase in stage of the tumours, which was statistically significant (p<0.00001).

Out of 6 Grade 1 tumours, all cases showed Ki-67 labelling index <15% and no cases showed Ki-67 labelling index ≥15%. Out of 16 grade 2 tumours, 15 cases showed Ki-67 labelling index <15% and 1 case showed Ki-67 labelling index ≥15%. Out of 12 grade 3 tumours, no cases showed Ki-67 labelling index <15% and all cases showed Ki-67 labelling index ≥15%. Out of 14 grade 4 tumours, no cases showed Ki-67 labelling index <15% and all cases showed Ki-67 labelling index ≥15%. So, it was noted that Ki-67 labelling index showed significantly increasing trend with the increase in histological grade of the tumours, which was statistically significant. (p<0.00001). (Table 2)

Correlation of VEGF Expression with Presence of LVSI and Necrosis:

Out of 10 VEGF grade 0 tumours, only one case showed presence of LVSI and one case showed presence of necrosis. Out of 21 VEGF grade 1 tumours, 8 cases showed presence of LVSI and 13 cases showed presence of necrosis. Out of 19 VEGF grade 2 tumours, LVSI was present in 13 cases and 17 cases showed presence of necrosis. So, we found significant association between VEGF grade and presence of LVSI and necrosis (p=0.0083). (Table 3)

Correlation and Comparison between VEGF and Ki-67:

Out of 23 tumours in which Ki-67 expression was <15%, 10 tumours were VEGF grade 0, 13 tumours were VEGF grade 1 and no tumours showed expression of VEGF as Grade 2. Out of 27 tumours in which Ki-67 expression was ≥15%, no tumours were VEGF grade 0, 8 tumours were VEGF grade 1 and 19 tumours showed expression of VEGF as grade 2. So, it was evident that the Ki-67 labelling index showed significantly increasing trend with the increase in VEGF grades of the tumours, which was statistically significant. (p<0.00001) (Table 4)

Tumour Stage	VEGF Grades			Total	p-Value
	0	1	2		
I	8 (80%)	1 (10%)	1 (10%)	10 (100.0%)	<0.00001
II	2 (14.3%)	8 (57.1%)	4 (28.6%)	14 (100.0%)	
III	0 (0%)	12 (48.0%)	13 (52.0%)	25 (100%)	
IV	0 (0%)	0 (0%)	1 (100.0%)	1 (100%)	
Tumour Grade					
1	4 (66.7%)	2 (33.3%)	0 (0.0%)	6 (100%)	<0.00001
2	5 (31.3%)	11 (68.8%)	0 (0.0%)	16 (100%)	
3	0 (0.0%)	5 (41.7%)	7 (58.3%)	12 (100%)	
4	0 (0.0%)	2 (14.3%)	12 (85.7%)	14 (100%)	

Table 1. Correlation of VEGF Grade with Stage and Histological Grade of RCC

Tumour Stage	Ki-67 Labelling Index		Total	p- Value
	<15%	≥15%		
I	9 (90.0%)	1 (10.0%)	10 (100.0%)	<0.00001
II	6 (42.9%)	8 (57.1%)	14 (100.0%)	
III	8 (32.0%)	17 (68.0%)	25 (100.0%)	
IV	0 (0.0%)	1 (100.0%)	1 (100.0%)	
Tumour Grade				
1	6 (100.0%)	0 (0.0%)	6 (100.0%)	<0.00001
2	15 (93.8%)	1 (6.3%)	16 (100.0%)	
3	0 (0.0%)	12 (100.0%)	12 (100.0%)	
4	0 (0.0%)	14 (100.0%)	14 (100.0%)	

Table 2. Correlation of Ki-67 Labelling Index with Stage and Histological Grade of RCCs

Necrosis	VEGF Grades			Total	p-Value
	0	1	2		
Present	1 (3.2%)	13 (41.9%)	17 (54.8%)	31 (100.0%)	0.0002
Absent	9 (47.4%)	8 (42.1%)	2 (10.5%)	19 (100.0%)	
Lymphovascular Invasion					
Present	1 (4.5%)	8 (36.4%)	13 (59.1%)	22 (100.0%)	0.0083
Absent	9 (32.1%)	13 (46.4%)	6 (21.4%)	28 (100.0%)	

Table 3. Correlation of VEGF Expression With Presence of Lymphovascular Invasion and Necrosis

Ki-67 Labelling Index	VEGF Grade			Total	p-Value
	0	1	2		
<15%	10 (43.5%)	13 (56.5%)	0 (0.0%)	23 (100.0%)	<0.0001
≥15%	0 (0.0%)	8 (29.6%)	19 (70.4%)	27 (100.0%)	

Table 4. Correlation of VEGF Grade with Ki-67 Labelling Index

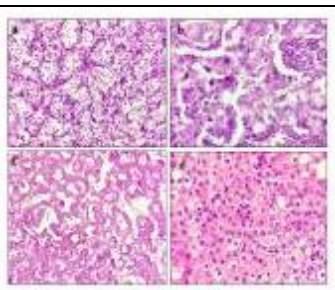

Figure 1. H&E photomicrographs of different subtypes of RCCs at 400X magnification: (a) Low Grade (Grade 1) Clear Cell Renal Cell Carcinoma (b) High Grade (Grade 4) Clear Cell Renal Cell Carcinoma (c) Papillary Renal Cell Carcinoma (d) Chromophobe Renal Cell Carcinoma

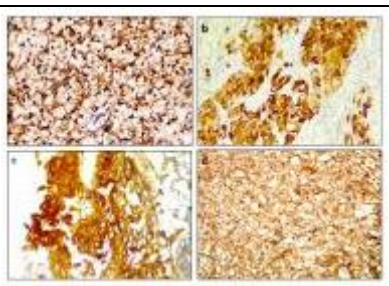

Figure 2. VEGF Expression in different Subtypes of RCC at 400X Magnification: (a) Low Grade (Grade 1) CCRCC Showing 1+ VEGF Positivity (2) High Grade (Grade 4) CCRCC showing 2+ VEGF Positivity (c) PRCC Showing 2+ VEGF Positivity (d) ChRCC Showing 1+ VEGF Positivity

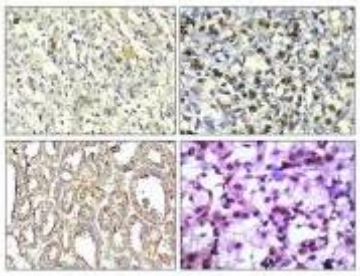

Figure 3. Ki-67 Expression in different Subtypes of RCC at 400X Magnification: (a) Low Grade (Grade 1) CCRCC Showing Ki-67 <15% (2) High Grade (Grade 4) CCRCC Showing Ki-67 ≥15% (c) PRCC Showing Ki-67 ≥15% (d) ChRCC Showing Ki-67 ≥15%

DISCUSSION

RCC is a well-recognized as a malignant tumour with an unpredictable clinical course. Patients with tumours showing same histological features can show a wide variation in biological behaviour and clinical outcome[17]. Renal cell carcinoma has the poorest prognosis among all the urological tumours. Tumour stage is the most powerful predictor of prognosis. Among histological parameters, WHO/ISUP nuclear grade is considered the most important prognostic parameter but it often shows substantial intra-observer and inter-observer variation.[15]

There are no current immunohistochemical prognostic markers which are routinely used for RCCs. In this era of new treatment possibilities, there is need for better prognostic tools to plan the treatment and follow-up of RCC patients. Proliferation index of RCC as determined by Ki-67 is known to have prognostic importance in univariate and multi-variate analysis and it also correlates with tumour grade.[15] In recent years importance has been given to the expression of different angiogenic factors like VEGF in RCCs.[18]

On this background, we conducted a study to assess VEGF expression and Ki-67 labelling index in RCCs and document the possibility of a correlation between these markers with different known prognostic parameters of RCC by IHC.

In our study, most of the RCC cases were VEGF as grade-1 (42.0%) while 38% cases were VEGF grade-2 and 20% were VEGF grade-0 or with no staining which corroborate with the findings of Ebru T et al[19] who got only 4 cases which did not show staining while 27 cases (37.5%) showed strong VEGF staining.

Yang S et al[20] found VEGF-A expression in 51.5% of RCC cases, which was significantly higher than the rate of expression in normal renal tissue surrounding the carcinoma. We found VEGF expression in 80% of RCC cases. This difference in expression levels may be attributed to diversity in detection techniques and varying sample size.

We also found a significant association of nuclear grade with VEGF expression. This was similar to the studies done by Yildiz E et al,[16] Bürgesser M et al[18] and Osman WM et al.[21]

Expression of VEGF grades was seen to increase with the increase in stage of the tumours. This observation was found to be concordant with Ebru T et al,[19] Bürgesser M et al[18] and Osman WM et al.[21]

No significant difference was found in our study between different RCC types concerning cytoplasmic VEGF expression which is concordant with findings of Yildiz E et al[16] and Matušan-Ilijaš K et al.[22]

Furthermore, we noted that VEGF grade showed significant association with presence of necrosis and lymphovascular invasion which is similar to studies by Fujita N et al[23] and Veselaj F et al.[24]

In our study, we found the mean Ki-67 labelling index (mean ± s.d.) of the patients was 14.32 ± 3.88 with a range 5 – 22 and the median was 15.0. Most of the tumours (54.0%) had Ki-67 labelling index ≥15% while the rest 46% of cases had Ki-67 labelling index<15%. In the study done by Amouian S et al,[25] out of 30 tumours studied, 20 (66.6%) were positive for Ki67. In the study done by Delahunt B et al,[26] 206 cases were studied, and Ki-67 expression was detected in 83%. Out of 1239 cases studied by Zheng K et al,[27] Ki-67 was detected in 47.7%. However, different authors have used different cut-off values for Ki-67 labelling index.

We also noted that Ki-67 proliferative index increased with increase in nuclear grade. This was similar to the studies done by Wong PK et al,[28] Bui MH et al,[29] Zheng K et al,[27] Amouian S et al[25] and Gelb AB et al,[30] who also found significant association of nuclear grade with Ki-67 labelling index.

In the studies done by Onda H et al,[31] Bürgesser M et al[18] and Gayed BA et al[32] respectively, Ki-67 expression was correlated with tumour stage which was concordant with our study.

We found no significant correlation between Ki-67 expression and histologic tumour subtypes. This was similar to the studies conducted by Wong PK et al[28] and Mehdi MZ et al.[15]

In current study, there was significant association between VEGF grades and Ki-67 labelling index of RCCs ($p<0.0001$). The Ki-67 labelling index showed significantly increasing trend with the increase in VEGF grades of the tumours which was concordant with Bürgesser M et al,[18] However, this finding was discordant with Matušan-Ilijaš K et al[22] who did not find any association between these markers.

CONCLUSION

Considering the observations of the current study, it can be concluded that the significant increase in VEGF expression and Ki-67 labelling index with tumour stage and grade and other prognostic parameters indicate that these two markers are associated with tumour growth and progression in RCCs. Their combined expression has a beneficial role in prediction of high stage tumours (Stage III/IV) and provides means for determining tumours that will respond to anti-angiogenic therapies. Since our study was limited by time, relatively small numbers of cases and minimum opportunity for follow up, further studies involving large number of cases with proper scope for follow up is needed to validate these results. Last but not the least, a better understanding of molecular pathways involved in pathogenesis and growth of tumours may help in the development of new strategies and target therapies for the early detection and treatment of RCCs.

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